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The combined extraction of sage (*Salvia officinalis* L.): Ultrasound followed by supercritical CO_2 extraction

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ABSTRACT

A wide spectrum of phytochemicals could be isolated from sage (*Salvia officinalis* L.) using different extraction or distillation technique: the supercritical fluid extraction (SFE), the volatiles compounds (monoterpenes and sesquiterpenes) isolation using hydrodistillation or higher molecular compounds with Soxhlet extraction or ultrasound-assisted extraction. The combination of ultrasound-assisted extraction followed by re-extraction of obtained extract with supercritical CO_2 was performed in this study. The goal of performed investigation was to concentrate diterpenes present in sage extract which are generally considered to be responsible for antioxidant activity of extracted compounds.

The fractionation using the supercritical CO_2, and different combination of the ultrasound-assisted solvent extractions (water–ethanol mixture or only water) followed by supercritical CO_2 re-extraction of obtained extract or treated plant material were analyzed and compared. Based on the results of these investigations it could be proposed the best extraction procedure: the ultrasound pretreatment of plant material with distilled water and re-extraction of plant material (residue) using supercritical CO_2. That procedure gives two valuable products: the ultrasound extract which is rich in sugars and possess the immunomodulatory activity and supercritical extract which is rich in diterpenes and sesquiterpenes.

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1. Introduction

Salvia officinalis L. (*Lamiaceae*) extract possesses specific characteristics which could be used as: antibacterial, hypoglycemic, anti-inflammatory, fungistatic, virustatic, astringent, eupeptic, anti-hydrotic and anti-diabetic products [1–10]. It is well known that extract from *S. officinalis* L. has a significant antioxidant [1–6] behavior which is used in food preservation or for medical/pharmaceutical care.

There are so many different extraction techniques described in literature for the sage extraction as, e.g.: the hydrodistillation, the conventional organic solvent extraction, the ultrasound-assisted solvent extraction and the subcritical or supercritical extraction using CO_2 as solvent (SFE or $SCCO_2$ extraction). All of them have an advantages and disadvantages with respect to operating cost, capital cost, yield, and quality of obtained extracts. Only the SFE possesses an environmental friendly and human health safety attributes. None of the above mentioned conventional techniques could be used alone for concentrating different groups of compound in the specific sage's product (extract). Most of these techniques are used for isolation either only the lighter fractions or sage's essential oil or total/crude extract (with heavy components as a waxes, oleoresins, sugars, chlorophyll, etc.). Among the many components present in the extract, the most valuable components of sage material are diterpenes possessing strong antioxidative behavior [11].

The $SCCO_2$ extraction could concentrate diterpenes in collected fractions or differential extracts by varying the temperature and pressure (or density) as was previously reported [1]. Regarding the total extract composition as well as content of isolated fraction it was found that 50 °C and 150 bar are the best operating conditions which gave the maximal selectivity of diterpenes [1].

In Eastern Europe herbal tinctures are often employed in medical treatment. Their production from conventional processes is not, however, very effective. A comparative study of the composition of the ethanol extract obtained from *Salvia officinalis* by conventional and ultrasound-assisted extraction indicates more efficient extraction using the latter method [12–15]. The use of ultrasound resulted in increased yield of extract in a shorter time and at lower temperatures [11].

The generally accepted explanation for ultrasound enhancement is related to the effect of ultrasonic waves on the plant material and breaks of cells and releases of cells' contents into the extraction medium. It is well known that an ultrasonic device is an adequate unit for the disruption of plant cells, but again no

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detailed information exists for the effect of ultrasound on plant tissues. Furthermore, the extraction from dried material could be described as a two-step process involving: (i) swelling and solvation of plant materials in corresponding solvent; (ii) the mass transfer of soluble constituents from the plant material to solvent by diffusion and osmosis [13].

The possible benefits of ultrasound application in extraction are most probably an intensification of mass transfer, improved solvent penetration into the plant tissue and capillary effects. All of these effects make an easier access of the solvent to the plant cell. The collapse of cavitation bubbles near the cell walls is expected to produce the cell disruption together with a good penetration of the solvent into the cells, through the ultrasonic jet. Indeed, there is direct evidence for the ultrasound effects on plant materials [13].

From a botanical standpoint, the plant tissues are composed of cells surrounded by middle lamella which consists of pectinous compounds, thus the plant cells are surrounded by membranes and the cell walls. In a live cell, the wall is a network of crystalline cellulose microfibrils glued together by various cross-linked polysaccharides, phenolic compounds and proteins into a coherent "matrix" that allows diffusion to take place. During the drying process of the plant tissues, a series of physical and chemical modifications take place, producing major differences between the fresh and the dried tissue. Thus, it is known that the dried cells lose their capacity for diffusion and osmosis as a result of cell wall and middle lamella desiccation. The rehydration process which leads to swelling during the steeping stage of extraction, is the result of the contact between the large number of hydroxyl groups, the cell wall and middle lamella. The general effect of ultrasonic treatment is to enhance the process of softening *via* the hydration of pectinous material from the middle lamella which then becomes more malleable. This leads to the break-up of the plant tissues with ultrasonic vibration in which the primary benefits of sonication during solvent extraction are an enhanced hydration process, which takes place simultaneously with plant (vegetal) material fragmentation [13].

The extract's yield depends on used solvent and increases with increasing its polarity. It is well known that the plant material contains hydrophilic and hydrophobic compounds but the content of the water soluble compounds is much higher.

The commercial and common procedure of sage products manufacturing are: essential oil isolation (hydrodistillation), preparation of different herbal tinctures (ethanol or ethanol/water extraction) and isolation of crude extract (SFE or $SCCO_2$). From the point of the full valorization of the plant material, hydrodistillation process has negative influence to the isolation of main antioxidant components because their degradation is much more expressed at boiling temperature of water. The insoluble sage residue represents somehow a waste material formed during the production of herbal tinctures. However it could be a source of polysaccharides which might possess biological activities, similar to those obtained with other medicinal plants.

The comparative analysis of combined extraction techniques (the ultrasound-assisted solvent extraction followed by, e.g. supercritical extraction) and supercritical extraction, which might fulfil the main task to obtain the extract with desired concentration of phenolic diterpenes is the main task of this study.

The objectives of this study were focused on diterpenes isolation applying combination of several extraction techniques: (a) the fractionation using the $SCCO_2$ signed as experiment A; (b) the ultrasound-assisted solvent (water–ethanol) extraction followed by $SCCO_2$ re-extraction of obtained extract (signed as experiment I); (c) the ultrasound water extraction followed by water–ethanol ultrasound-assisted extraction of residue and finalized by $SCCO_2$ re-extraction of extract (signed as experiment II), and (d) the ultrasound water extraction followed by $SCCO_2$ re-extraction of residue, i.e. plant material (signed as experiment III).

2. Material and methods

2.1. Materials

Dalmatian sage (*Salvia* officinalis L.) was collected in September/October 2008 (late flowering stage) from the region Konavle (the very southern tip of Croatia) and the voucher specimen (No. 16,282) was deposited at Herbarium of Institute of Botany and Botanical Garden Jevremovac, Faculty of Biology, University of Belgrade (Belgrade, Serbia). Aerial parts of sage (leaves to plant ratio = 70:30) were dried in the air protected against direct sunlight. Leaves were milled in a blender (model MMB1000, 500 W, Robert Bosch Hausgeratte GMbh, Munich, Germany) for 60 s and immediately subjected to supercritical CO_2 extraction, or ultrasound-assisted extraction as a first step which is used before supercritical CO_2 extraction. The average particle size of milled sage was 0.55 mm. The moisture content of the air-dried plant material determined by Karl Fischer volumetric titration (Karl Fisher titrator DL18, Mettler-Toledo Inc., Columbus, USA) was 13.6 mass%.

Commercial carbon dioxide (99% purity) was supplied by Tehnogas (Messer-Tehnogas, Serbia), 95% ethanol (Zorka Pharma, Serbia) for Soxhlet extraction. The standard compounds used for the chemical analyses were carnosic acid and carnosol (Sigma Chemical Co. (St. Louis, USA)). These chemicals were of analytical reagent grade.

2.2. Equipment and methods

2.2.1. Ultrasound-assisted extraction with different solvents

The milled plant material (50 g) was added to an Erlenmeyer flask and extracted with different solvents using different mass/volume of plant material and solvent (1:12 or 1:6) for 45 min at 40 ± 1 °C using the ultrasound bath. Ultrasound-assisted extraction was performed in an ultrasound cleaning bath (Sonorex, RK 52 type, with power of 600 W, 140 × 135 × 100 mm internal dimensions, BANDELIN electronic GmbH & Co. KG, Munich, Germany) by the mode of the indirect sonication, at the fixed-frequency of 35 kHz using as the working liquid. Different solvents were used for ultrasound extraction and preparation of extract for further step of $SCCO_2$ extraction: experiment I – water and ethanol in mass proportion 30:70; experiment II – two steps realized using distilled water and then residue or plant material was treated by water and ethanol mixture (Table 1). Experiment III was realized by ultrasound extraction with distilled water followed by $SCCO_2$ extraction of residue (plant material) (Table 1). At the end of experiment I the liquid extract was mixed with a fixed amount (3:1 mass ratio to extract) of glass particles (2 mm size) and evaporated to the dryness by rotary vacuum evaporator at 50 °C. After solvent evaporation (vacuum evaporator, Devarot, Elektomedicina, Ljubljana, Yugoslavia), each glass particle was coated with a thin film of crude oleoresin. Glass particles coated with crude oleoresin were placed as a bed into the extractor of the high pressure system for further extraction. At the end of II extraction the liquid extract was finally treated by $SCCO_2$ extraction. All extractions' procedures were performed in triplicate with 0.7% standard deviation.

2.2.2. Supercritical carbon dioxide extraction

Extraction with supercritical CO_2 (experiment A, as well as the final step of experiments I, II and III) was carried out in a semi-batch Autoclave Engineers Screening System (Autoclave Engineers,

Table 1
The summarized information of experimental procedure.

Experiment	Pretreatment with ultrasound (at 40 °C for 45 min)	$SCCO_2$ fractionation/extraction at 50 °C and 150 bar	Labeling of samples
A	No	Extraction from plant material 8 fractions (8th fraction collected at 300 bar)	FA1–FA8
I	Ethanol–water (70:30) and 1:12 m/v plant material to solvent	Re-extraction using extract (4 fractions were collected)	FI1–FI4
II	1. Water and 1:6 m/v ratio of plant material to solvent 2. Ethanol–water (70:30) and 1:12 m/v ratio of plant material to solvent	Re-extraction from extract (3 fractions were collected)	FII1–FII3
III	Distilled water and 1:6 m/v ratio of plant material to solvent	Extraction from residue of plant material	SC extract

Erie, Pennsylvania, USA) previously described in details [1,16]. All the supercritical CO_2 extraction's experiments were performed isothermally at 50 °C and at 150 bar which is optimal condition for the diterpenes selectivity based on results of previous study [1]. Several SFEs (re-extraction process) were realized using 30 g of milled plant material as well as using determined amount of extracts in the case of experiments I and II or plant residue in the experiment III. Also, the extraction at 300 bar and 50 °C was performed with the aim to obtain the total extract [1] by supercritical CO_2 and for a further comparison with the results of the other experiments realized in this study. The flow rate of CO_2 was 0.4 kg h^{-1} in all runs. Differential quantities of extract (fractions) were collected during the extraction with main goal to monitor the changes of the chemical composition of the extract with respect to CO_2 consumption and pressure. The standard deviation of measured yield (triplicate procedure) was 2.3% for all experiments. All yields and composition calculations were made on a moisture basis (moisture of 13.6 mass%).

The obtained extracts were also kept in a sealed vial at 4 °C prepared for GC–MS analyses. The detailed experimental procedure is presented in Section 3. Results of this article and detailed experimental procedure are summarized in Table 1.

2.3. Analytical procedures

2.3.1. Analytical gas chromatography (GC/FID)

Gas-chromatography analysis of the extracts was carried out on a HP-5890 Series II GC apparatus (Hewlett-Packard, Waldbronn, Germany), equipped with split–splitless injector and automatic liquid sampler, attached to HP-5 column (25 m × 0.32 mm, 0.52 μm film thickness) and fitted to flame ionization detector (FID). Carrier gas flow rate (H_2) was 1 ml/min, split ratio 1:30, injector temperature was 250 °C, detector temperature 300 °C, while the column temperature was linearly increased (40–260 °C; 4 °C/min), and then kept isothermally at 260 °C for 10 min. Solutions of samples in ethanol (or mixture of ethanol and chloroform, 50:50 in the case when the extract was partially insoluble in ethanol) (~1 mass%) were consecutively injected in amount of 1 μl. Area percent reports, obtained as result of standard processing of chromatograms, were used as base for the quantification analysis.

2.3.2. Gas chromatography/mass spectrometry (GC/MS)

The same analytical conditions as those mentioned for GC/FID were employed for GC/MS analysis (HP-5MS column: 30 m × 0.25 mm, 0.25 μm film thickness), using HP G 1800C Series II GCD system [Hewlett-Packard, Palo Alto, CA (USA)]. Helium was used as carrier gas. Transfer line was heated at 260 °C. Mass spectra were acquired in EI mode (70 eV); in m/z range 40–450. The amount of 0.2 μl of sample solution in ethanol (or mixture of ethanol and chloroform, 50:50) (~1 mass%) was injected.

The components of the extract were identified by comparison of their mass spectra to those from Wiley 275 and NIST/NBS libraries, using different search engines. The experimental values for retention indices were determined by the use of calibrated Automated Mass Spectral Deconvolution and Identification System Software (AMDIS ver.2.1.) [17], compared to those from available literature [18], and used as additional tool to approve MS findings. The percentage composition of each volatile compound and fatty acids methyl esters was the mean value of three replicates (±standard deviation, SD).

3. Results

Major compounds in sage extract and the content of different compound families such as monoterpenes (M), oxygenated monoterpenes (OM), sesquiterpenes (S), oxygenated sesquiterpenes (OS), diterpenes (D), triterpenes (T), esters (E) and waxes (W) identified by means of GC–FID and GC–MS methods are listed in Table 2 (average result of cumulative extract collected at specific condition used for extraction).

Sage's extract obtained by ultrasound extraction (water–ethanol) mainly contains the groups of diterpenes (29.91%), triterpenes (34.13%) and waxes (14.43%), while $SCCO_2$ extraction at 150 and 300 bars gave: monoterpenes (5.81–6.12%), oxygenated monoterpenes (44.95–48.21%), sesquiterpenes (3.87–4.08%), oxygenated sesquiterpenes (7.10–8.93%), diterpenes (23.98–28.60%), esters (2.30–2.36%), triterpenes (2.55–3.66%) and waxes (3.83–3.87%) (Table 2). The oxygenated monoterpenes were the most abundant in the SFE extract along with diterpenes and oxygenated sesquiterpenes, and the major compounds were manool, β-thujone, carnosol derivatives, camphor and viridoflorol. In this study carnosol derivatives were identified instead of carnosic acid and carnosol which are commonly found in sage extract due to their thermal degradation during gas-chromatography analysis [19].

The hypothetical composition of the sage plant material used in this study was calculated and proposed (Table 3 and the last column of Table 2 for the total extract composition). Based on this it could be concluded that ultrasound-assisted solvent extraction provided the method which enabled the highest yield of sage extract (14.21 mass%), while supercritical extraction at 300 bar resulted in 4.65 mass% yield of extract. The solvent extraction provided isolation of diterpenes in a higher amount but it was followed by extraction at the same time of other higher molecular compound such as triterpenes and waxes (Table 2). The supercritical CO_2 extraction (even at 300 bar) is less selective for waxes and oleoresins than solvent extraction, and thus containing diterpenes in larger percentages, is more valuable extract. Also, it could be seen from Tables 2 and 3 that by using the ultrasound-assisted solvent extraction (followed with vacuum evaporation of solvent) the easily evaporated compounds (mainly monoterpenes and oxygenated monoterpenes) were lost.

3.1. Fractionation with supercritical CO_2 – experiment A

Fractional extraction of sage at specific condition of $SCCO_2$ enables concentrating of lower molecular compounds (monoterpenes and oxygenated monoterpenes) followed by minimal co-extraction of higher molecular compounds. Supercritical extraction

Table 2
The major compounds identified in different sage extracts.

Components	KI	RT/MS	SD	US[a] EtOH + H_2O	SFE 300 bar	SFE 150 bar	In plant material
				g/100 g of plant material			
Monoterpenes				0.01	0.27	0.24	0.28
α-Pinene	934	7.40	0.01	0.00	0.10	0.09	0.10
Camphene	949	7.84	0.03	0.00	0.07	0.06	0.07
β-pinene	977	8.73	0.01	0.00	0.02	0.02	0.02
Myrcene	995	9.30	0.15	0.00	0.01	0.01	0.01
p-Cymene	1027	10.37	0.21	0.00	0.01	0.01	0.01
Limonene	1031	10.50	0.02	0.00	0.02	0.01	0.02
γ-Terpinene	1061	11.56	0.26	0.00	0.04	0.03	0.04
Trans-sabinene hydrate	1101	12.93	0.01	0.00	0.01	0.01	0.01
Oxygenated monoterpenes				0.25	2.09	1.89	2.34
1,8-Cineole	1033	10.57	0.02	0.00	0.23	0.17	0.23
β-Thujone	1109	13.21	0.02	0.04	0.60	0.62	0.64
α-Thujone	1119	13.57	0.02	0.01	0.39	0.29	0.40
Iso-3-thujanol	1138	14.22	0.02	0.00	0.01	0.01	0.01
Trans-pinocarveol	1140	14.32	0.02	0.00	0.01	0.01	0.01
Camphor	1146	14.52	0.01	0.04	0.51	0.48	0.55
Neo-3-thujanol	1155	14.80	0.01	0.00	0.11	0.09	0.11
Borneol	1169	15.30	0.01	0.03	0.02	0.01	0.05
Terpinen-4-ol	1181	15.74	0.05	0.00	0.00	0.00	0.00
α-Terpineol	1198	16.31	0.04	0.00	0.01	0.01	0.01
Myrtenol	1202	16.46	0.03	0.01	0.03	0.03	0.04
Bornyl acetate	1289	19.41	0.02	0.01	0.04	0.03	0.05
α-Campholenic acid	1346	21.28	0.02	0.07	0.10	0.09	0.17
Acetophloroglucine[b]	1354	21.52	0.02	0.01	0.03	0.03	0.04
Sesquiterpenes				0.17	0.18	0.16	0.35
Cis-α-bergamotene	1418	23.55	0.02	0.09	0.01	0.01	0.10
Trans-caryophyllene	1422	23.68	0.02	0.04	0.07	0.05	0.11
α-Humulene	1456	24.74	0.01	0.04	0.09	0.10	0.13
Oxygenated sesquiterpenes				0.24	0.33	0.35	0.57
Caryophyllene oxide	1585	28.61	0.31	0.02	0.03	0.03	0.05
Viridiflorol	1594	28.88	0.17	0.15	0.20	0.22	0.35
Humulene epoxide I	1599	29.02	0.03	0.00	0.01	0.01	0.01
Humulene epoxide ii	1611	29.36	0.03	0.05	0.06	0.06	0.11
Muurola-4.10(14)-dien-1-β-ol	1634	29.98	0.02	0.01	0.02	0.03	0.03
14-Hydroxy-cis-caryophyllene	1660	30.69	0.02	0.00	0.01	0.01	0.01
Diterpenes				1.03	1.33	0.94	2.36
Manool	2059	40.68	0.15	0.45	0.81	0.70	1.26
Carnosol derivative[b]			0.15	0.53	0.45	0.24	0.98
Trans-ferruginol	2330	46.56	0.15	0.05	0.07	0.00	0.12
Ester derivatives				0.07	0.11	0.09	0.18
Methyl hexadecanoate	2096	41.53	0.01	0.04	0.04	0.03	0.08
Methyl undecanoate	2103	41.68	0.01	0.00	0.01	0.01	0.01
Methyl oleate	2130	42.28	0.01	0.03	0.05	0.04	0.08
Methyl octadecanoate	2150	43.57	0.01	0.00	0.01	0.00	0.01
Waxes				0.50	0.18	0.15	0.68
Heptacosane	2731	54.45	0.01	0.04	0.04	0.02	0.08
Octacosane	2879	57.43	0.01	0.00	0.07	0.06	0.07
Nonacosane	3031	60.40	0.03	0.21	0.03	0.01	0.24
Untriacontane	3115	62.13	0.02	0.24	0.05	0.05	0.29
Triterpenes				1.18	0.17	0.10	1.35
t-Sitosterol[a]	2812	56.10	0.01	0.04	0.02	0.01	0.06
Olean-18-ene	2798	55.82	0.01	0.86	0.11	0.07	0.97
Lupeol	2900	57.85	0.01	0.28	0.04	0.03	0.32
Total, g/100 g of plant material				3.45	4.65	3.92	8.11

KI – kovats index; RT/MS – retention time of corresponding constituent obtained by GC/MS; SD – standard deviation
[a] Water insoluble extract obtained after ultrasound treatment of plant material (the first step of experiment I).
[b] Tentative identification.

performed at 150 bar followed by higher CO_2 consumption (>37 $kg_{CO_2}/kg_{plant\ material}$) is favourable for concentrating the higher molar compounds in extract. The average selectivity of diterpenes compared to heavier compounds (triterpenes, esters and waxes) was the highest at 150 bar due to lower solubility of triterpenes, esters and waxes in CO_2 at noted pressure [1].

The differential selectivity of diterpenes compared to monoterpenes and sesquiterpenes, as well as differential selectivity of diterpenes compared to heavier compounds (esters, triterpenes and waxes) was the highest at 150 bar. It is the result of a very small amount of other heavier compounds present in extract isolated using carbon dioxide at 150 bar (Fig. 1). Presence of the carnosol derivatives even in the first fractions (FA1, Fig. 1) collected at 150 bar can be explained by carnosic acid solubility in CO_2 even at lower pressure (lower CO_2 density) [1]. Namely, it was found that up to 300 bar only carnosic acid is soluble in CO_2, while above 300 bar solubility of carnosol starts to increase significantly as well [1].

Table 3
Composition of sage plant material.

Components	Composition in sage plant material (mass% per dried plant)	
Other plant material (cellulose)	49.9	
Humidity	13.6	
Sugars	26.0	
Chlorophyll	2.4	
Extract (containing 0.7 mass% of essential oil)	8.1	
	Gram in 100 g of plant material	Mass% in extract
Monoterpenes	0.28	3.51
Oxygenated monoterpenes	2.34	28.79
Sesquiterpenes	0.35	4.34
Oxygenated sesquiterpenes	0.57	7.00
Diterpenes	2.36	29.12
Ester derivatives	0.18	2.25
Waxes	0.68	8.36
Triterpenes	1.35	16.62
Total of terpenes	8.11	100.00
Total	100	

This study indicated that CO_2 consumption at 150 bar and 50 °C which is between 3 and 10 $kg_{CO_2}/kg_{plant\ material}$ favors the isolation of lower molar mass compounds (monoterpenes and oxygenated monoterpenes). A longer extraction time and extraction under higher pressure (300 bars and more) seemed to be favorable for extraction of compounds with higher molar mass. Increased solubility of compounds with a higher molar mass at 300 bar $SCCO_2$ extraction and thus increase of extract's yield is evidently followed by decrease of diterpenes selectivity. Detailed analysis of resistance to diffusion of heavier compound throughout the structure of plant material must also be taken into the account for explanation of diterpenes selectivity at specific condition.

Differential fractions were collected during $SCCO_2$ extraction at 50 °C and 150 bar and one of them contains up to 62 mass% of diterpenes (detected in F8A, Fig. 1). However, the amount of this fraction is very small comparing to the total amount of collected extract (up to 2 mass%). Specific fraction containing a higher amount of carnosol could be only isolated under higher pressure (>300 bar) when carnosol starts to be more soluble in supercritical CO_2.

First four fractions (FA1–FA4 representing the 68.2 mass% of the total extract) have the same composition and they are rich of monoterpenes. Such composition profile was expected due to similar solubility of their constituents in supercritical CO_2. According to these results the fractionation with supercritical CO_2 could be used for concentrating diterpenes in several fractions (up to 39.7 mass%) but theoretically they represent less than 25% of total mass of extract according to the average composition of sage (Tables 2 and 3). Namely, experimentally determination of the mass of fractions FA1–FA8 at 50 °C and 150 bar takes only 16 mass% of total amount which might be isolated from sage.

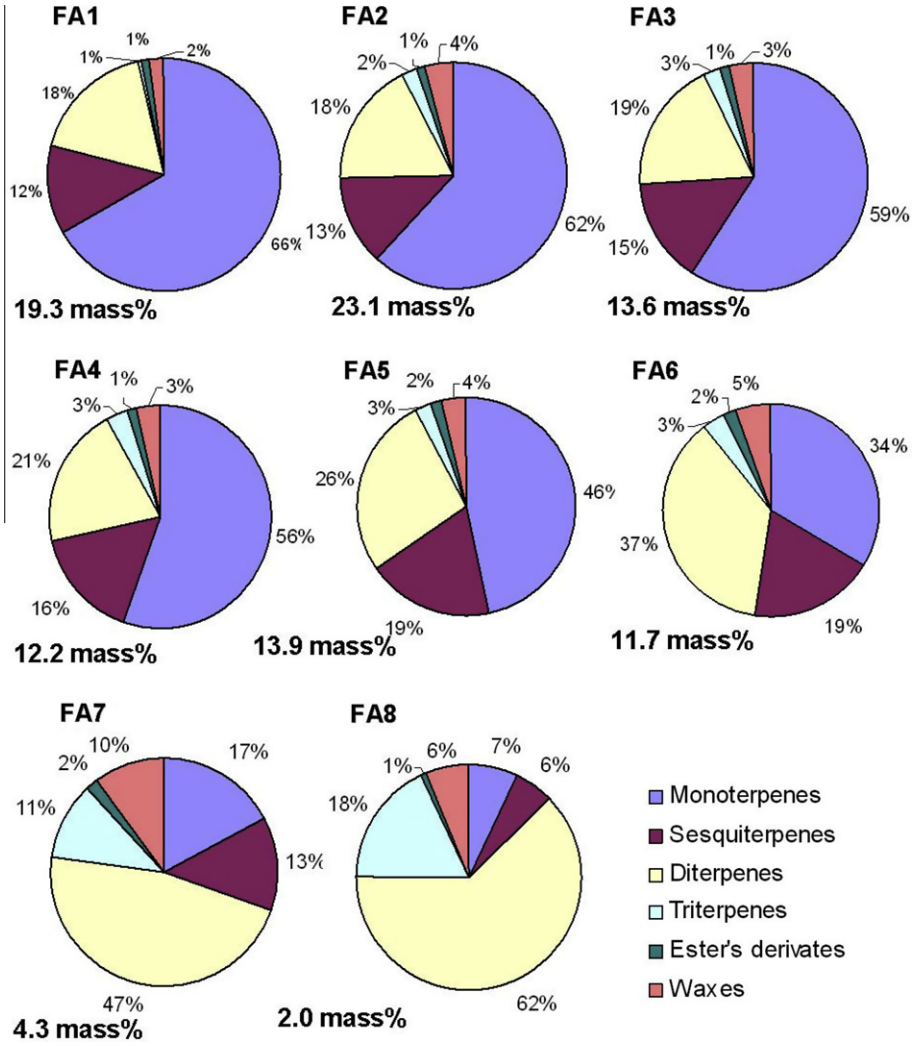

Fig. 1. The chemical composition of obtained fraction during fractionation with CO_2 at 150 bar and 50 °C.

3.2. Combined the ultrasound-assisted solvent extraction and $SCCO_2$ fractionation

3.2.1. Experiment I

The ultrasound-assisted extraction was performed at 40 °C using ethanol–water mixture (70:30) and 18 mass% yield of extract after 45 min of treatment was detected (Fig. 2). Re-extraction of obtained extract was performed using supercritical CO_2 at 50 °C and 150 bar and collected re-extract was separated into 4 fractions (FI1–FI4). The yield of deodorized sage re-extract was 6.0 mass% (with respect to the mass of ultrasonically obtained extract) or 1.08 mass% with respect to the initial mass of plant material used for combined process of extraction. The complete experimental procedure and corresponding mass balance of experiment I are shown on Fig. 2.

The composition of FI1–FI4 fractions of sage re-extract was determined by GC/FID and GC/MS analysis and the results are presented in Table 4.

Differences in composition profiles of FI1–FI4 fractions, i.e. in total re-extract and extract obtained from sage (*Salvia officinalis* L.) performing only by supercritical CO_2 extraction at 50 °C and 150 bars seemed that combined technique is effective for diterpenes isolation. Namely, the content of diterpenes in sage re-extract is higher (about 61 mass%) compared to the content of diterpenes in essential oil obtained by hydrodistillation (1.5 mass%, [1]), the Soxhlet extraction (about 30 mass%) or SFE extraction of sage plant material at 50 °C and 150 bars (about 24 mass%). Also, the lower content of aromatic and volatile compounds evidently exists compared to the content of ultrasound extract (Table 2) or fractions obtained by SFE at 50 °C and 150 bars (FA1–FA8, Fig. 1). However, significantly smaller total amount of re-extract could be probably explained by incorporation of specific compounds present in extract into the crystal structure of polysaccharides.

3.2.2. Experiment II

Based on the result of yield of combined water–ethanol ultrasound extraction and re-extraction of extract with supercritical

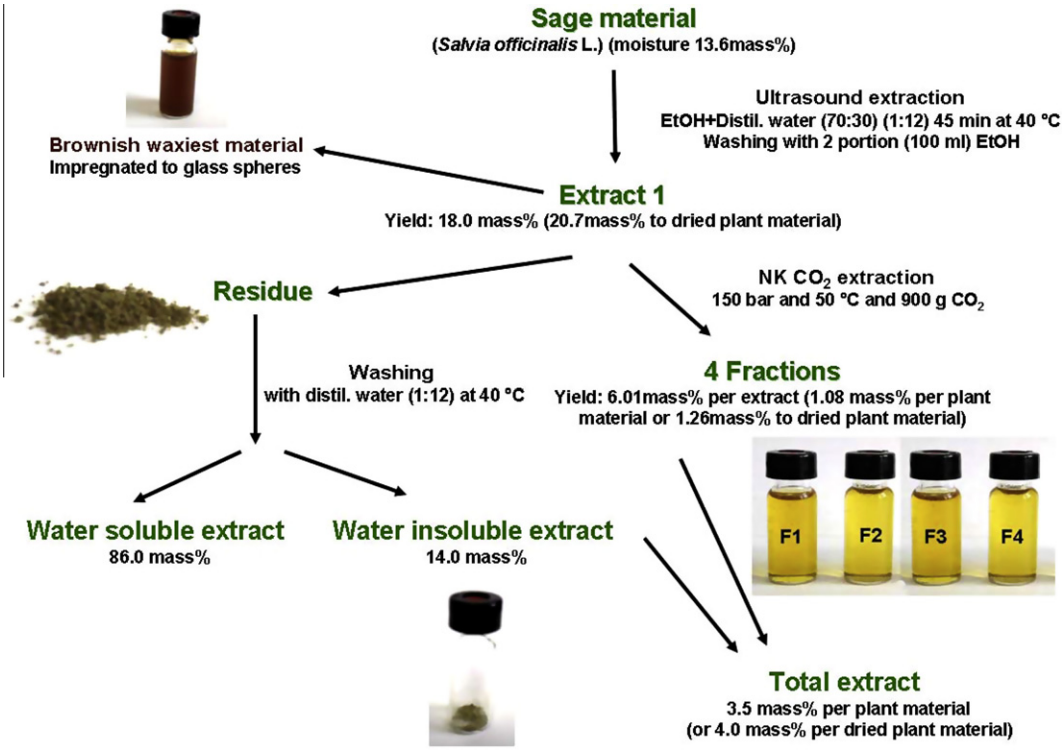

Fig. 2. The schematic presentation of the experiment I: ultrasound-assisted water–ethanol extraction and re-extraction of the obtained extract at 150 bar and 50 °C.

Table 4
The chemical composition of extract and fractions (re-extract) obtained in experiment I.

Components	Composition, mass%						
	Extract	Residue in extractor	Average in FI1-FI4 fractions	Fractions of re-extract			
				FI1	FI2	FI3	FI4
Monoterpenes	0.25	0.25	0.96	0.60	0.55	0.50	0.25
Oxygenated monoterpenes	4.24	4.21	8.65	9.59	8.11	7.13	4.24
Sesquiterpenes	2.97	3.19	1.92	2.58	2.21	2.02	2.97
Oxygenated sesquiterpenes	6.90	6.48	20.19	21.13	18.42	16.95	6.9
Diterpenes	29.85	30.45	48.08	48.52	46.70	48.94	29.85
Ester derivates	2.02	1.48	12.50	9.42	13.20	14.28	2.02
Waxes	14.36	15.78	5.77	4.10	6.94	4.57	14.36
Triterpenes	34.02	34.02	1.92	0.00	0.21	2.56	34.02
Total mass, %	94.61	94.61	100	95.93	96.34	96.94	94.61
Total mass, g	17.03	15.99	1.04	0.38	0.36	0.22	0.08

CO_2, the next procedure of combined technique of extraction (II) was realized with the main goal at first to remove polysaccharides from the ultrasonically obtained extract. So, the first step in ultrasound-assisted extraction was performed at 40 °C using distilled water and it was yielded by 22.5 mass% of extract after 45 min of treatment (Table 1 and Fig. 3). The treated plant material (residue) was after that extracted ultrasonically using ethanol–water mixture (70:30) at 40 °C and 6.3 mass% yield of extract after 45 min

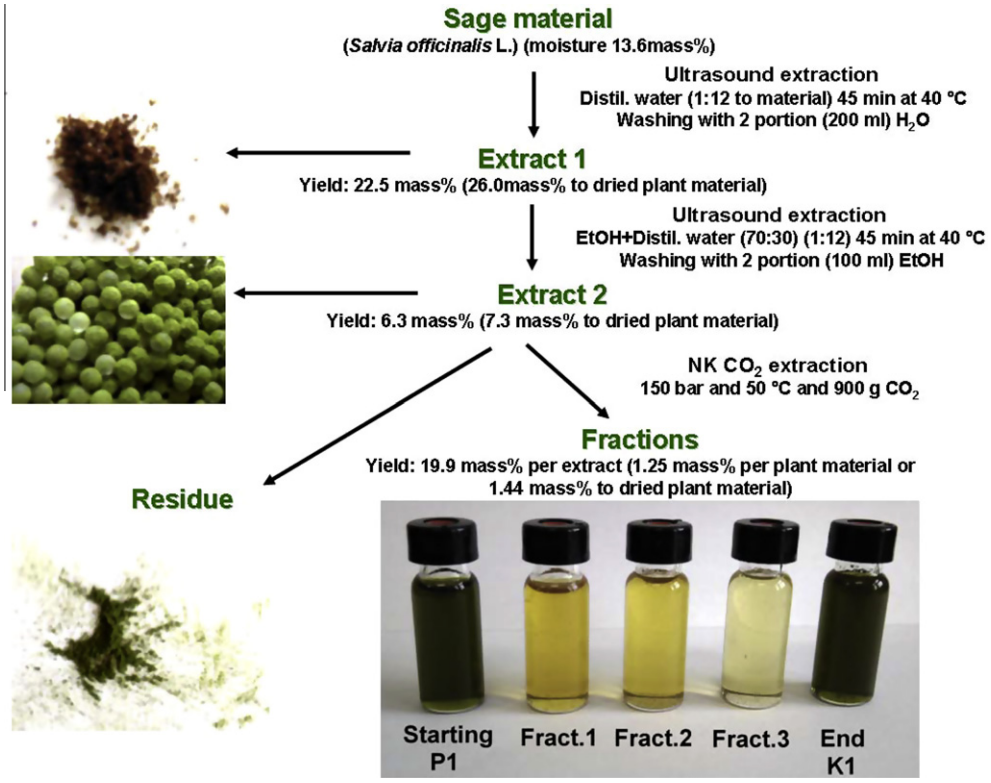

Fig. 3. The schematic presentation of the experiment II: ultrasound-assisted water followed by ultrasound water–ethanol extraction and finally re-extraction of obtained extract at 150 bar and 50 °C.

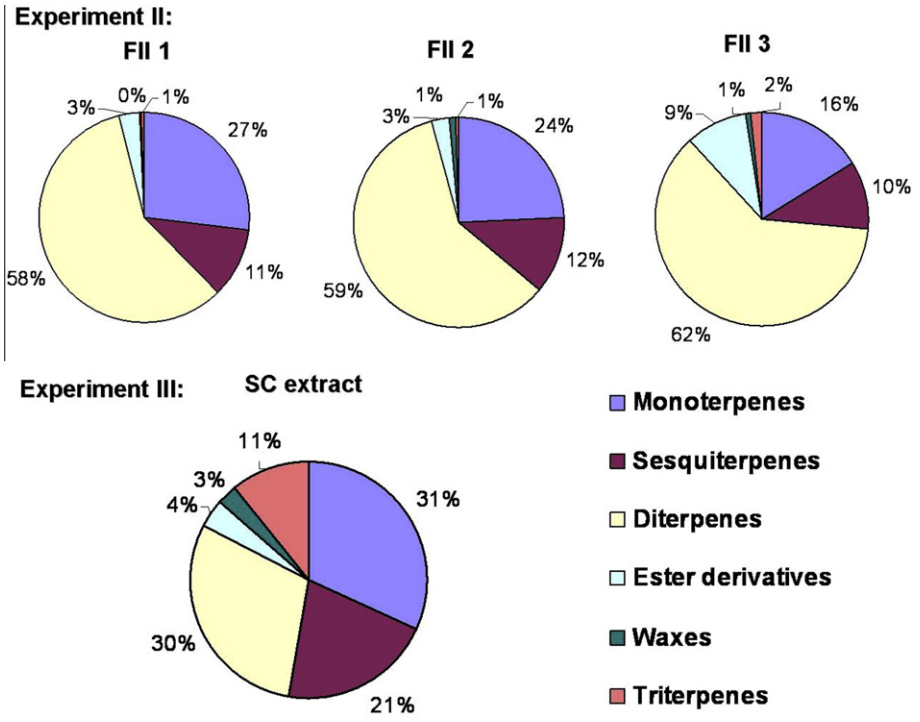

Fig. 4. The chemical composition of fractions (experiment II) and re-extract obtained at experiment III with $SCCO_2$ at 150 bar and 50 °C.

of treatment was detected. Obtained extract, after the evaporation in vacuum was re-extracted with supercritical CO_2 at 50 °C and 150 bars and 3 fractions were collected (FII1–FII3). The yield of deodorized sage re-extract was 19.9 mass% (with respect to the mass of ultrasonically obtained extract, Fig. 3) or 1.25 mass% with respect to the initial mass of plant material used for combined process of ultrasound extraction – $SCCO_2$ re-extraction (experiment II). The detailed experimental procedure and corresponding mass balance performed in experiment II are shown in Fig. 3.

The obtained results indicated that the removal of polysaccharides in the first step of combined extraction technique did not solve indicated problem in the previous experiment (I). Namely, only 33.3 mass% of total amount of extract could be re-extracted using $SCCO_2$ what means that the major part and the possible valuable part of extract still remains in the residue after re-extraction. These results are similar to the previous one obtained in experiment I. Although, the removal of polysaccharides by distilled water pretreatment increases the yield of re-extract for 15%, the co-extracted material during the ultrasound-assisted treatment (probably chlorophyll, based on the color of ultrasound extract) still suppresses the extraction of heavier terpenes. The composition of fractions collected in the process of supercritical CO_2 re-extraction is shown in Fig. 4. It could be seen that the content of diterpenes increases in fractions and takes the 58–62 mass% which is quite better then values obtained in previous experiments (up to 50 mass%).

3.2.3. Experiment– III

The ultrasound-assisted extraction was performed at 40 °C using distilled water with the aim to remove the water soluble components, mainly polysaccharides, from the plant material. The obtained yield of extract was 22.4 mass% after 45 min of treatment. The water insoluble components, i.e. the valuable heavier compounds remain in the plant material. The residue of plant material was dried at the room temperature for 12 h and after that determined humidity of sage was 41.3 mass%. Prepared plant material was extracted with supercritical CO_2 at 50 °C and 150 bars and the yield of sage extract (SC extract) was 2.11 mass% calculated to the plant material or 3.59 mass% calculated to the mass of dried sage.

The composition profile of obtained SC extract is shown in Fig. 4. The SC re-extract (experiment III) and total extract obtained using $SCCO_2$ extraction without pretreatment show significantly different composition profile (Table 2 and Fig. 4). Namely, the water soluble (small amount of waxes) was extracted together with polysaccharides while the easily evaporated components (mainly monoterpenes and oxygenated monoterpenes) were loosed during the drying procedure of plant material residue. The $SCCO_2$ extract has a 5% higher content of diterpenes (regarding the extract obtained without pretreatment) but the content of sesquiterpenes is 45% higher and triterpenes is 3.6 times more. This result could be explained by the cell wall disruption of plant material by ultrasound and accessibility of components with higher molar mass to be in contact with $SCCO_2$.

4. Discussion

The combination of two different extraction techniques was performed having a goal to enhance the yield of extract, as well as, to concentrate diterpenes in supercritical extract. Namely, it could be concluded that the $SCCO_2$ performed at 300 bar and 50 °C yielded the maximum of valuable extract from the plant material [1]. The other techniques could only extract a lighter fractions, e.g. hydrodistillation (the yield is 0.7 mass% of essential oil [1]) or total/crude extract using Soxlet or ultrasonic assisted solvent extraction (the yield is 22 mass% [1], containing heavy components as waxes, oleoresins, sugars, chlorophyll, etc.). Furthermore, detailed analyses of the chemical composition of the extract and re-extract show that the complete valuable extract (rich in terpenes, oleoresins and esters) could be obtained only with $SCCO_2$ extraction.

The $SCCO_2$ extraction at 150 bar and 50 °C is the best extraction procedure for obtaining the highest selectivity of diterpenes in extract [1]; however, the highest yield could be obtained at 300 bar and 50 °C where concentration of diterpenes in extract is about 28.6 mass%.

The extraction curves (yields versus CO_2 consumption for extraction or re-extraction) of all performed experiments in this study (A, I–III) are presented in Fig. 5.

The fractionation with supercritical CO_2 (FA1–FA8; 150 bar and 50 °C) could concentrate diterpenes up to 39.7 mass% in fractions compared to 24 mass% to the total SC CO_2 extract (Table 2 and Fig. 1). However, the mass of all fractions (FA1–FA8) represents only 16 mass% of extract.

The combined techniques (experiments I and II) could be used with aim to concentrate diterpenes up to 50 mass% in re-extract but the total mass of collected fractions representing re-extract gave only 33 mass% of the total possible $SCCO_2$ extract at 150 bar and 50 °C. As could be seen, the yields of re-extraction from the extract obtained with ultrasound and different solvents (experiments I and II) are quite similar also giving the similar composition profile of the obtained re-extracts or their fractions (FI1–FI4 and FII1–FII3). The $SCCO_2$ re-extraction from the plant material previously treated with distilled water and ultrasound (experiment III) shows the similar extraction curve as that obtained with $SCCO_2$ extraction up to consumption of 7 $g_{CO_2}/g_{\text{plant material}}$. Extraction followed by larger CO_2 consumption (>7 $g_{CO2}/g_{\text{plant material}}$) resulted by 17.1% smaller yield of re-extract (experiment III) comparing to the yield of extract obtained in experiment A. It is caused by losses of monoterpenes during the first step or preparation treatment of experiment III.

Summarizing all obtained results and derived conclusions; it could be proposed the best extraction procedure: pretreatment of plant material with distilled water and ultrasound and further re-extraction of plant material (residue) using $SCCO_2$. That procedure gives two valuable products: a) water extract – rich in sugars

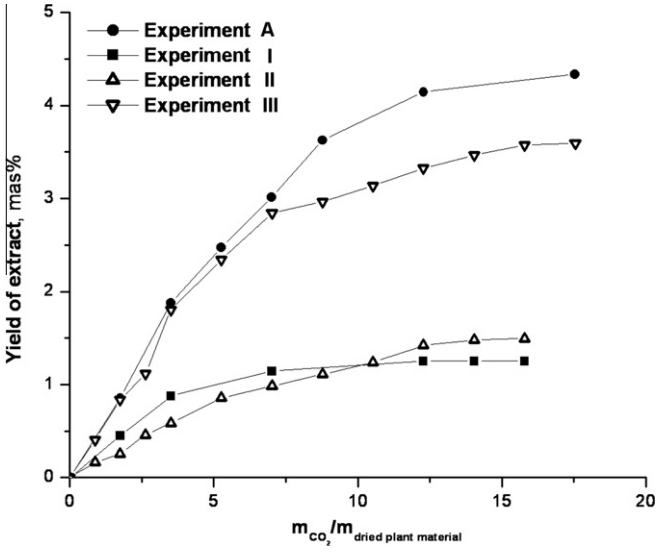

Fig. 5. The extraction curves for all experiments (the yield of extract in experiment A and re-extracts in experiments I–III calculated to the mass of the dried plant material).

which possess the immunomodulatory activity [20]; and, b) extract – rich in diterpenes (up to 30 mass%) and sesquiterpenes.

5. Conclusion

The analysis of combined technique of extraction in series, performed in this study, had a goal to show that compounds of diterpenes type could be concentrated in final product.

The ultrasound-assisted extraction was performed at 40 °C using ethanol–water mixture (70:30) without (experiment I) and with ultrasound pretreatment of plant material with distilled water (45 min; 40 °C; experiment II). The yields of extracts were after ultrasoundly assisted extractions: 20.7 mass% based on dry plant in experiment I and, 26 mass% of ultrasound water pretreatment followed by 7.3 mass% yield after ethanol–water mixture (70:30) ultrasound extraction. In both cases (I and II) the collected extracts were further separated into several fractions using supercritical CO_2 re-extraction at 50 °C and 150 bar. It was shown that the sage re-extracts (I and II) had a lower yield and higher content of diterpenes compared to the extract obtained by SFE sage extraction alone (experiment A) at 50 °C and 150 bar. However, it was also shown that combined procedure, the supercritical CO_2 extraction at 50 °C and 150 bar of the previously treated plant material using only distilled water assisted with ultrasound (experiment III) could give at the same time two valuable products: the water extract and diterpenes' concentrated extract.

Acknowledgements

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